American Journal of Pathology, Vol. 137, No. 6, December 1990

Reversibility of Hepatic Fibrosis in Experimentally Induced Cholestasis in Rat

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The reversibility of hepatic fibrosis was investigated in an experimental model of extrahepatic cholestasis in the rat after common bile duct ligation for 2 weeks, followed by bilioduodenal anastomosis for 3 weeks. Bile duct ligation resulted in a transitory marked elevation in the serum concentration of 5'-nucleotidase, alkaline phosphatase, and bilirubin during the first 3 days. Then these levels decreased to threefold, twofold, and 100-fold the normal values, respectively, during the following 4 weeks. Histologic examination of the liver disclosed extensive bile duct proliferation and the formation of periportal fibrosis, with only slight inflammation and necrosis. The distribution of the major components of the hepatic extracellular matrix was analyzed 2 weeks after bile duct ligation, using the indirect immunoperoxidase method. Fibrous septa were found to be strongly stained for collagens I, pro-III, III and IV, fibronectin, and laminin. The most intense staining was found in enlarged periportal areas, collagen IV and laminin being particularly abundant around newly formed bile ducts. These changes paralleled high steady-state levels of α_1(I) and α_1(IV) collagen and B_2 chain laminin mRNAs. Relief of the obstruction for 2 weeks resulted in a shift in the serum concentration of 5'-nucleotidase, alkaline phosphatase, and bilirubin toward normal values. A dramatic resorption of bile duct proliferations and periportal fibrosis were observed. Three weeks after bile duct repermeabilization, immunohistochemical study showed that the pattern of distribution of extracellular matrix components was almost normal, except for collagen IV, which remained abundant in the sinusoids when compared with the normal liver. In parallel, the steady-state B_2-chain laminin mRNA level became lower than in cholestatic livers, whereas α_1 (I) and α_1 (IV) mRNAs were almost undetectable. These results show that hepatic fibrosis induced by experimental extrahepatic cholestasis in rat disappears in less than 3 weeks after relief of bile duct obstruction, suggesting that an active degradation of matrix proteins occurs, except for collagen IV in the sinusoid. (Am J Pathol 1990, 137:1333–1342)

Hepatic fibrosis, which is one feature of liver cirrhosis, occurs in a variety of chronic liver diseases.[1] It consists primarily of increased deposition of extracellular matrix macromolecules, mainly collagens, noncollagenous glycoproteins, and proteoglycans.[2] Fibrogenesis is a complex process that involves alterations in both the synthesis and degradation of matrix proteins by different liver cell types. It is generally accepted that once it is established, fibrosis is irreversible.[1] A few observations, however, in man[3–5] and studies with animals have led to the hypothesis that experimental hepatic fibrosis is a reversible process, after discontinuation of the causative agents. Animal models have been proposed for studying the reversibility of hepatic fibrosis. These include *Schistosomiasis mansoni* in mice,[6–12] and chemicals, eg, CCl_4 and ethionine,[6,8,13] or high- or low-fat protein diets in rats.[14,15] Duration of the treatment and the difficulty of identifying the causes of fibrosis, however, had made these models matter in debate. The most commonly used method of producing experimental cirrhosis involves multiple doses of CCl_4. This toxic agent rapidly induces liver damage and severely alters metabolism and gene expression of hepatocytes.[16] Chronic CCl_4 intoxication results in extensive necrosis of parenchymal cells and inflammation.

Extrahepatic cholestasis is another model to investigate the formation of hepatic fibrosis. Prolonged obstruction of bile flow or hepatic diseases that lead to anatomic destruction of the biliary tree results in morphologic and bio-

Supported by INSERM. Pierre-Yves Rescan was a recipient of a fellowship from La Ligue Française contre le Cancer.

Accepted for publication July 23, 1990.

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chemical changes and the development of secondary biliary cirrhosis.[17] Changes induced by experimental bile duct ligation in the rat have been partially analyzed.[18,19] They include an extensive proliferation of bile ducts in enlarged portal spaces, with slight inflammation and necrosis, and the formation of periportal fibrosis in less than 2 weeks after obstruction of the biliary tree.[20] The molecular mechanisms of cholestasis-induced fibrosis, however, are yet unknown. In addition, the possibility that cholestasis-induced fibrosis is a reversible phenomenon has not been explored.

We have studied the formation and the reversibility of fibrosis in rat liver with experimentally induced cholestasis. We show that restoration of the normal biliary outflow in 2-week-old bile duct-ligated rats results in a complete reversion of fibrosis with a nearly normal distribution of the major components of extracellular matrix, including collagens and the two noncollagenous glycoproteins, laminin and fibronectin, and a parallel shift in the steady-state level of the corresponding mRNAs.

Material and Methods

Antisera and cDNA Clones

Antisera against human plasma fibronectin and collagens from fibrotic human livers (types I and III), calf skin (type pro-III), and bovine lens capsule (type IV) were provided by Dr. J. A. Grimaud (Institut Pasteur, Lyon, France). Antisera against laminin, extracted from the Engelbreth-Holm-Swarm tumor, were a gift from Dr D. Louvard (Institut Pasteur, Paris). These antisera have been shown previously to recognize antigens from rat origin.[21-23]

All cDNA probes coding for extracellular matrix proteins were of murine origin. Laminin B_2 cDNA probe, a gift from Dr Y. Yamada (NIDR, Bethesda, MD), was a 1.5 kb *Eco*R1–*Hind* III fragment of P7 cloned from a F9 cell cDNA library.[24] Collagen α1 (I) and α1 (IV) cDNA probes were from Dr. M. Laurent (INSERM U 118, Paris, France). They were, respectively, a 0.97-kb PST I fragment coding for the entire C-terminal propeptide region and a part of the helicoidal region, and a 0.65 kb PST I fragment coding for a part of the helicoidal region.[25] β-Actin and albumin cDNA probes were from rat origin.[26,27]

Animals

Male Sprague-Dawley rats weighing 180 to 200 g were used. They were divided in five groups and underwent the following procedure: group A (n = 10), common bile duct ligation; group B (n = 8), sham-operated rats, control of group A; group C (n = 12), bilioduodenal anastomosis for 21 days after common bile duct ligation for 14 days; group D (n = 8), sham-operated rats, control of group C; group E (n = 4), normal rats. Peripheral blood samples were routinely assayed for total bilirubin, alkaline phosphatase, and 5′-nucleotidase, using standard laboratory techniques on a COBAS Bio apparatus (Roche, Switzerland).

Fixation and Immunohistochemical Procedure

The livers were washed through the portal vein with phosphate-buffered saline (PBS) (Na_2HPO_4 12 H_2O, 8 mmol/l [millimolar], NaH_2PO_4, 1.9 mmol/l; NaCl, 138 mmol/l), pH 7.4, for 2 minutes, then perfused with a 4% paraformaldehyde solution buffered with 0.1 mol/l sodium cacodylate, pH 7.4, for 15 minutes at a flow rate of 10 ml/minute.[28] Liver fragments were cut and immediately immersed in PBS or postfixed in the same fixative for 4 hours at 4°C. These postfixed samples were embedded in paraffin and used for histologic examination. Sections were routinely stained with hemalun-eosin safran and Masson's trichrome. Other fragments were soaked for 60 minutes in PBS containing 10% glycerol before freezing in liquid nitrogen-cooled isopentane.

Extracellular matrix proteins were localized *in situ* using the indirect immunoperoxidase technique.[28] Briefly, 8-μ–thick cryostat sections were incubated in PBS containing 10% fetal calf serum and 0.1% saponin for 60 minutes. The sections were incubated in a dilution of specific antibodies in PBS containing 0.1% saponin for 1 hour. The second incubation was performed with peroxidase-labeled anti-immunoglobulins (Institut Pasteur, Paris) diluted in PBS containing 0.1% saponin. Then, sections were postfixed in 2.5% glutaraldehyde buffered with 0.1 mol/l sodium cacodylate for 4 minutes at 4°C, followed by incubation in 300 mmol/l glycine, pH 10, for 15 minutes, to prevent nonspecific oxidation of the diaminobenzidine used to detect peroxidase activity. Peroxidase activity was shown by incubating sections in a 3.3′-diamino-benzidine/H_2O_2 solution for 20 minutes. Controls included a first incubation with nonimmune sera.

Isolation of RNA and Northern-blot Analyses

Total RNA was extracted from liver biopsies by the guanidinium-thiocyanate/cesium chloride method.[29] Total RNAs (10 μg) were subjected to electrophoresis in a denaturing 1.1 mol/l formaldehyde-agarose gel and transferred onto Hybond-N sheets (Amersham, Arlington

Heights, IL). Filters were soaked in hybridization buffer containing 3 × SSC, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% bovine serum albumin, 0.1% SDS, and 10% dextran sulfate, and then hybridized with the same solution, containing ^{32}P-labeled probes.

Results

Biochemical and Morphologic Changes

Common serum indices of cholestasis were measured in cholestatic rats (group A) and in repermeabilized rats (group C) and compared with those of sham-operated rats (groups B and D) (Figure 1). Only animals showing typical changes in enzyme concentrations indicative of liver cholestasis[30] were subsequently analyzed (n = 7 in group A; n = 8 in group C). Bilirubin, alkaline phosphatase, and 5′-nucleotidase peaked 3 days after common bile duct ligation, reaching 200-, 4- and 8-fold the normal values, respectively. During the following 4 weeks, alkaline phosphatase and 5′-nucleotidase activities decreased to a level about twofold and threefold the normal value, respectively. Bilirubin concentration decreased by day 14, and then slowly increased up to 100-fold the normal values on day 35. In rats undergoing bilioduodenal anastomosis for 24 days after 14 days' bile duct ligation, bilirubin, alkaline phosphatase, and 5′-nucleotidase rapidly shifted toward normal values during the first week after restoration of bile outflow. These values remained stable during the following 2 weeks.

Histologic liver changes were analyzed after 1 and 2 weeks' obstruction of the common bile duct and 1, 2, and 3 weeks after bilioduodenal anastomosis (Figure 2). Biliary obstruction resulted in rapid extensive bile duct proliferation and fibrogenesis in enlarged portal areas. Infiltration of the portal tract with polymorphonuclear inflammatory cells was occasional and hepatocyte necrosis was nearly absent. Central vein areas remained unchanged. When bilioduodenal anastomosis was performed 2 weeks after bile duct ligation, newly formed bile ducts and extensive portal fibrosis rapidly disappeared during the following 2 weeks. A nearly normal histology was observed 3 weeks after anastomosis.

Immunolocalization of Collagens, Fibronectin, and Laminin

In normal rat liver, the distribution of collagens I, pro-III, III, and IV, fibronectin, and laminin was similar to that previously described.[22,28] The major sites of deposition were portal spaces and, to a lesser extent, central veins (Figure 3). Collagens I and IV were the less abundant. In the sinusoids, fibronectin formed a continuous layer and collagen pro-III was the most abundant type of collagen, although discontinuous. Basement membrane components, ie, collagen IV and laminin, were undetectable. Hepatocytes were intracellularly stained for fibronectin, whereas nonparenchymal cells located in the sinusoids were positive for laminin and, particularly in periportal areas, for collagen IV.

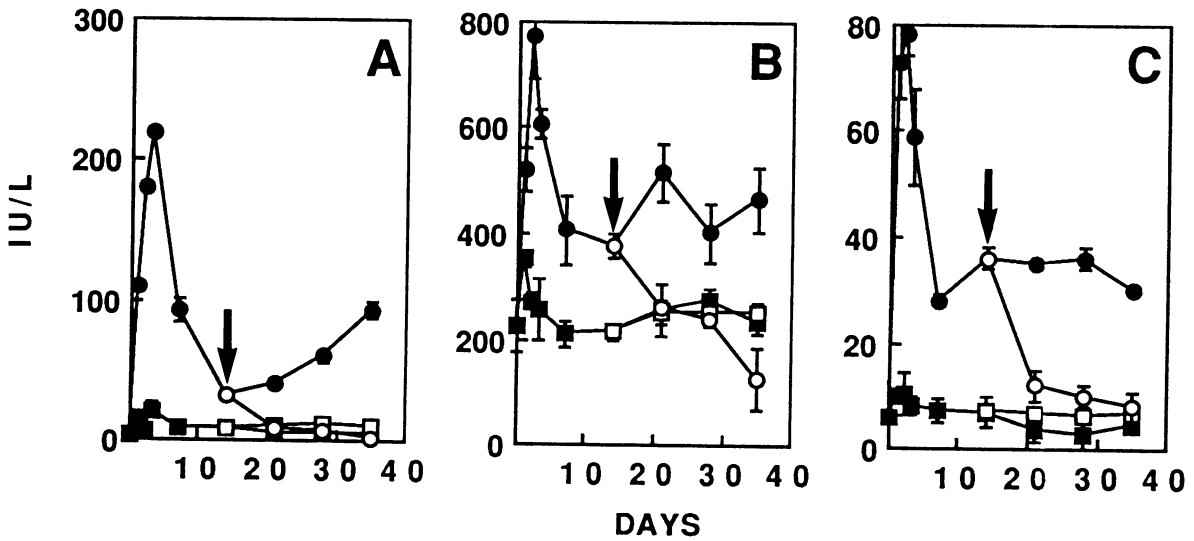

Figure 1. *Effects of biliary obstruction (day 0) and bilioduodenal anastomosis (day 14, arrow) on the serum concentration of bilirubin* (A), *alkaline phosphatase* (B), *and 5′-nucleotidase* (C). -●-: *common bile duct ligation (group A);* -○-: *common bile duct ligation for 14 days, followed by bilioduodenal anastomosis for 21 days (group C);* -■-: *sham-operated rats, control of group A (group B);* -□-: *sham-operated rats, control of group C (group D). Values are the mean ± SD.*

Fourteen days after common bile duct ligation, extensive fibrous septa were stained for collagens I, pro-III, III and IV, fibronectin, and laminin. The most intense staining was found in portal spaces. Intense staining for collagen IV and laminin was observed around bile duct proliferations. In the sinusoids, collagen IV was continuously distributed along hepatocyte cords. Laminin and fibronectin were deposited mainly within the periportal areas and in the borders of septa.

After the additional 21-day period that followed bilioduodenal anastomosis, the distribution of collagens, fibronectin, and laminin was in some extent similar to that observed in normal liver. In portal spaces, the remaining clusters of neo-formed bile ducts were still surrounded by abundant laminin and collagen IV. In the sinusoids, the distribution of collagens I, pro-III and III, laminin and fibronectin was similar to that in normal liver. Only collagen IV remained abundant in the sinusoids and continuously deposited all along hepatocyte cords.

These data show that cholestasis affects the distribution of all the major extracellular matrix components. After repermeabilization of the biliary tree, these recover a normal pattern except for collagen IV, which is still much more abundant in the sinusoid.

Collagens α_1 (I) and α_1 (IV) and Laminin B_2 mRNA Content

To investigate whether changes in extracellular matrix deposition in cholestatic rat liver are the result of alteration in matrix protein gene expression, the steady-state α_1 (I) and α_1 (IV) collagens, and laminin B_2 mRNA levels were studied by Northern blot in liver biopsies from bile duct-ligated rats, before and after bilioduodenal anastomosis, and compared with the steady-state β-actin and albumin mRNA levels (Figure 4). In normal rat liver, the α_1 (I), and α_1 (IV) collagens and laminin B_2 chain mRNAs were detectable only after a long-time exposure (up to 1 week) (data not shown), while β-actin and albumin mRNAs were abundant. After a 2-week obstruction of the common bile duct, the steady-state α_1 (I) and α_1 (IV) collagens and laminin B_2 chain mRNA levels were strongly increased, while copious amounts of β-actin mRNAs were found. Albumin mRNAs were considerably reduced.

Twenty-one days after bilioduodenal anastomosis, the pattern of mRNA content was almost that found in normal liver, with α_1 (I) and α_1 (IV) collagen mRNAs being barely detectable. Laminin B_2 mRNA level also decreased, but remained higher than in normal liver. A strong decrease was found for the β-actin mRNA content, which dropped to a lower level than in normal liver. The steady-state albumin mRNA content was similar to that found in normal liver. These results show that cholestasis-induced fibrosis is correlated with an increase in the content of matrix protein mRNAs. After restoration of the normal bile outflow, the decrease in matrix content paralleled that of matrix protein mRNAs.

Discussion

The reversibility of hepatic fibrosis remains the subject of controversy. We chose to investigate fibrogenesis in cholestatic rat liver experimentally induced by common bile duct ligation. This model is morphologically characterized by marked proliferation of bile ducts and formation of enlarged fibrous septa with only limited inflammation and necrosis. Dramatic and early increase in the serum concentration of bilirubin, alkaline phosphatase, and 5'-nucleotidase indicated that extrahepatic cholestasis was induced rapidly in this experimental model.[19,30] Our immunohistochemistry study demonstrates that fibrosis is formed by interstitial collagens (types I and III), fibronectin, and basement membrane proteins. These findings were confirmed by Northern-blot analysis of liver samples. α_1 (I)-interstitial and α_1 (IV)-basement membrane collagens mRNA levels were much higher in cholestatic rats than in normal rats. A similar observation was made for laminin B_2 chain mRNAs, thus showing that both collagens and noncollagenous glycoprotein genes were simultaneously overexpressed in cholestatic rat liver. As bile duct cells actively proliferated, it can be expected that they were the producers of basement membrane components, ie, collagen IV and laminin,[22,31] and responsible for the accumulation of these proteins in portal areas. Conversely, high levels of α_1 (I) collagen could be limited to normally producing cells or resulted from the recruitment of new producing-cell populations. It has been shown that the expression of matrix proteins in liver cells depends on their functional state and a variety of environmental factors.[32–37] For example, Ito cells, which synthesize collagens and laminin in the normal liver,[21,22] exhibit phenotypic alterations in culture. They have been shown to contain more interstitial collagen mRNAs in culture than immediately after isolation.[38] Hepatocytes also may express laminin or collagens in injured liver after alcoholic intoxication in man[22,36] or CCl_4 treatment in rat,[32] as well as in primary culture.[22,34,35] Immunofluorescence study has shown the presence of collagen IV in hepatocytes after a 8-day obstruction of the common bile duct in rat, suggesting that cholestasis may induce changes in the cellular sources of extracellular matrix components.[34] These findings are in agreement with our immunoelectron microscopy study showing that various liver cell types, including periportal

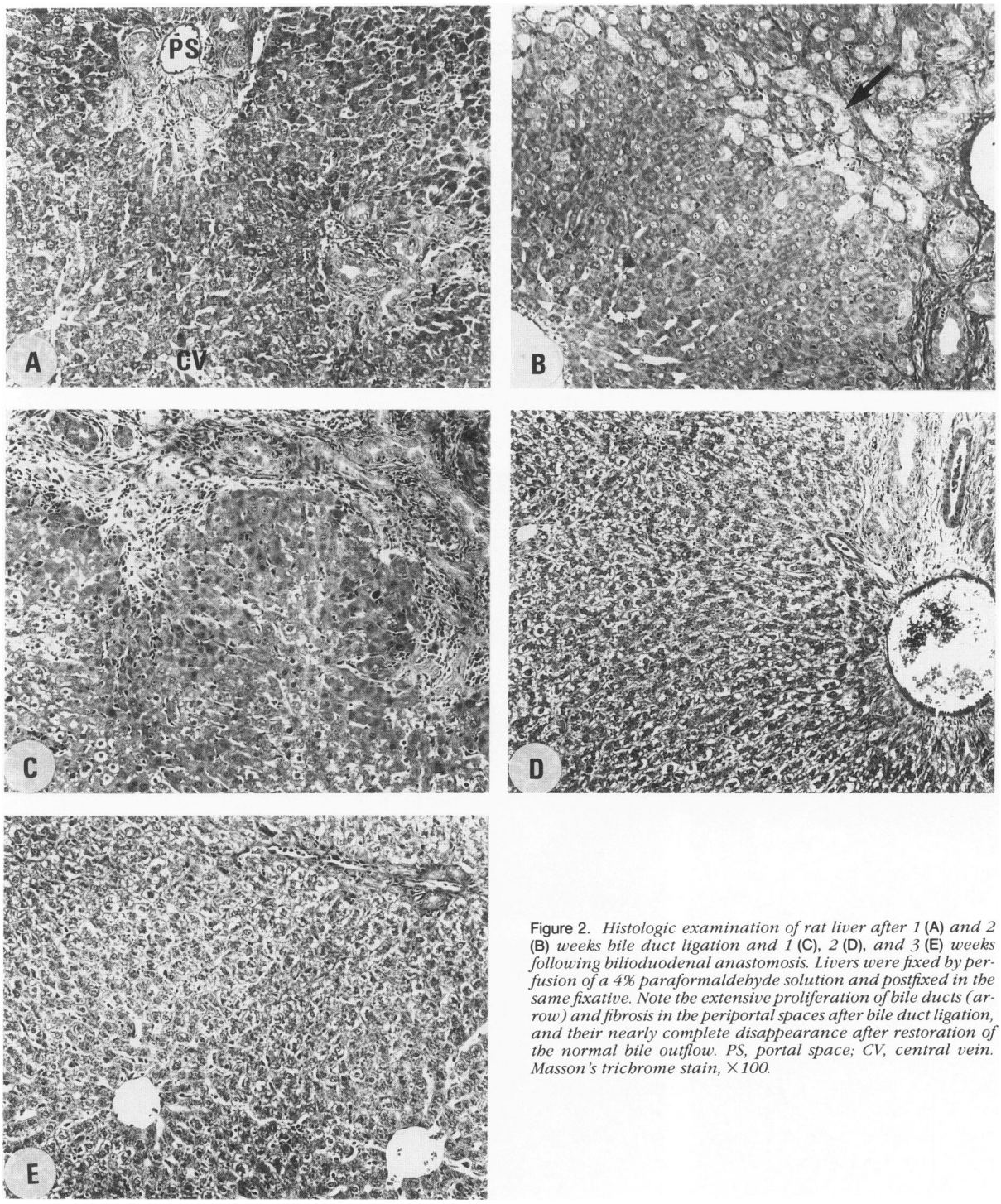

Figure 2. *Histologic examination of rat liver after 1* (A) *and 2* (B) *weeks bile duct ligation and 1* (C), *2* (D), *and 3* (E) *weeks following bilioduodenal anastomosis. Livers were fixed by perfusion of a 4% paraformaldehyde solution and postfixed in the same fixative. Note the extensive proliferation of bile ducts (arrow) and fibrosis in the periportal spaces after bile duct ligation, and their nearly complete disappearance after restoration of the normal bile outflow. PS, portal space; CV, central vein. Masson's trichrome stain, ×100.*

hepatocytes, participate in the formation of extracellular matrix proteins after ligation of the common bile duct[39].

A dramatic increase in the total β-actin mRNA content rapidly occurred in rat liver after common bile duct ligation. This is probably related to both the marked proliferation of bile duct cells in portal spaces and alteration in the distribution of cytoskeleton components in the hepatocytes. Cholestasis is known to induce changes in the pattern of actin microfilament distribution in parenchymal cells, particularly beneath bile canaliculus domains.[40,41]

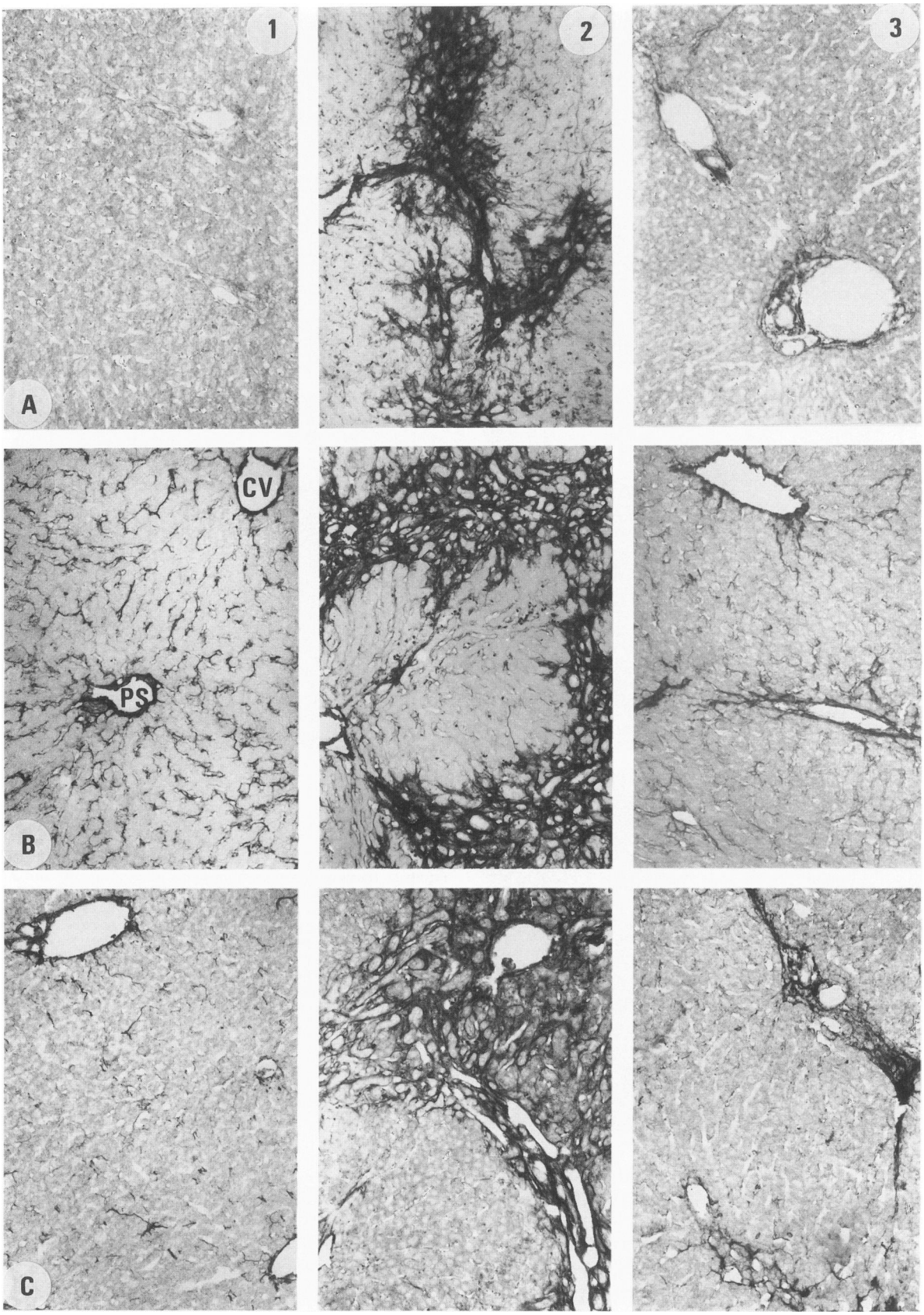

Figure 3. *Light microscopic immunolocalization of collagens I* (**A**), *pro-III* (**B**), *III* (**C**), *IV* (**D**), *laminin* (**E**), *and fibronectin* (**F**) *in normal rat liver (1), and in rat livers after a 2-week bile duct ligation (2) and following bilioduodenal anastomosis for 3 weeks (3).* ×110.

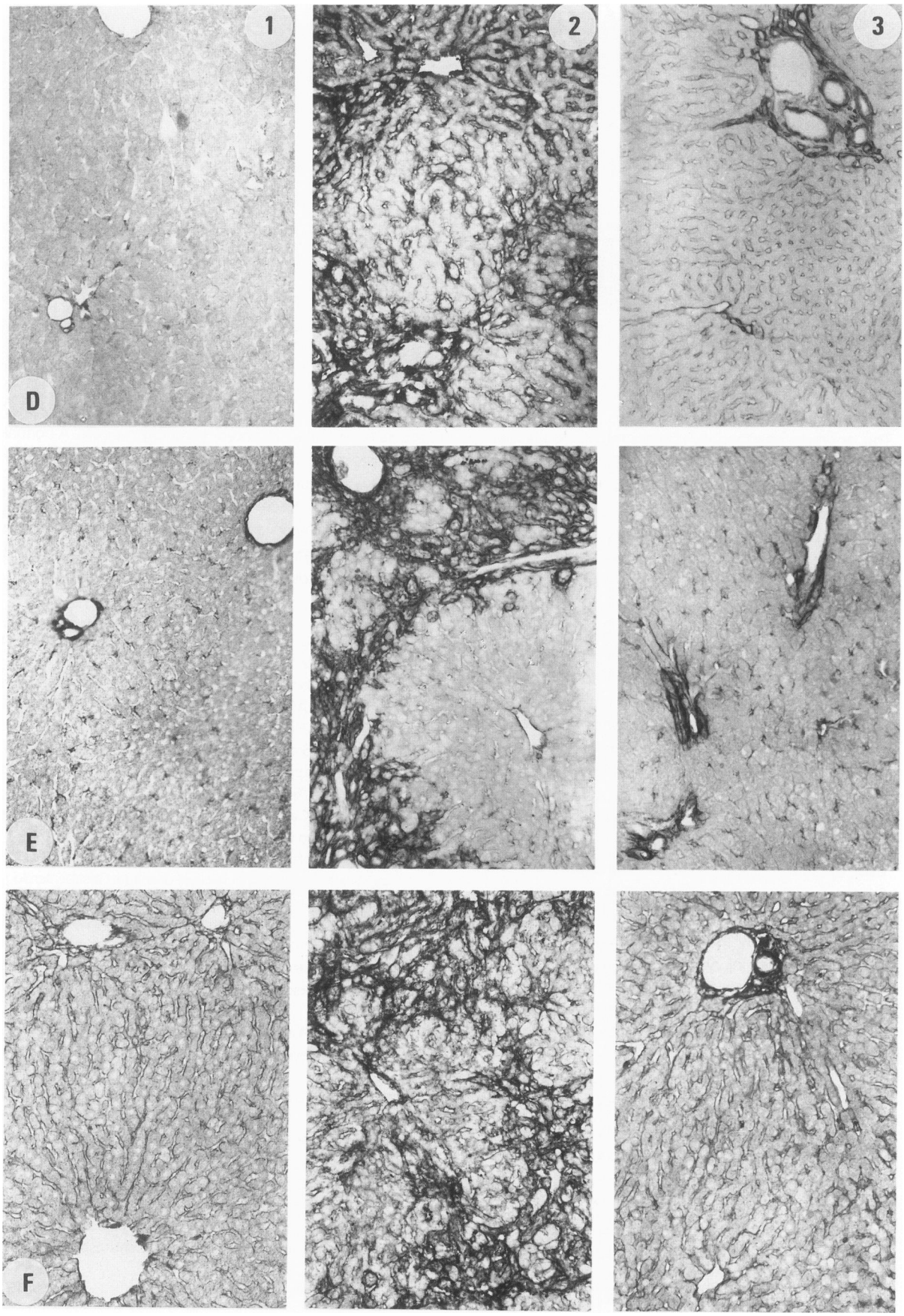

Figure 3. *(Continued).*

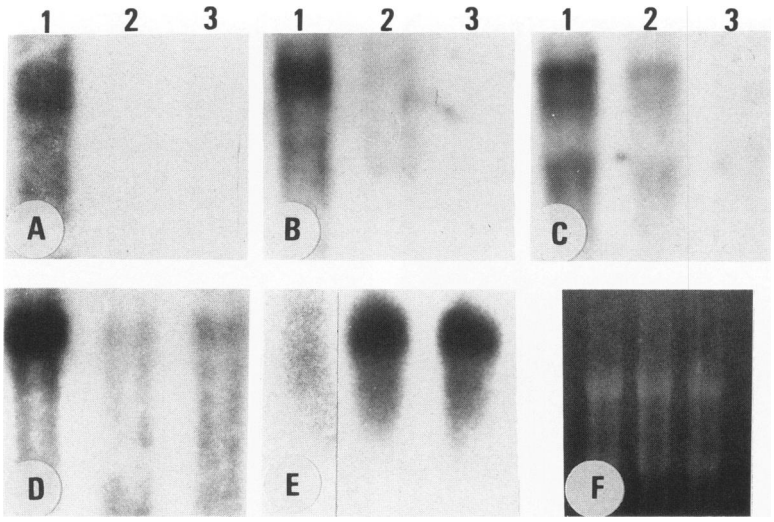

Figure 4. *Steady-state levels of α_1 (I) collagen* (A), *α_1 (IV) collagen* (B), *laminin B_2* (C) *β-actin* (D) *and albumin* (E) *mRNAs in rat livers 14 days after common bile duct ligation (1) and 21 days after bilioduodenal anastomosis (2), compared to normal liver (3). The amounts of total electrophoresed RNA (10 μg) were monitored by staining the gel with ethidium bromide (F) before transfer on nitrocellulose filters.*

The opposite changes in albumin mRNA levels clearly indicate that the increase in matrix protein gene expression is a specific response to cholestasis induced by bile duct ligation.

Bilioduodenal anastomosis resulted in a rapid shift to normal liver architecture and functions, including normal values in serum enzyme activities. Our histochemical study shows a nearly complete shift in periportal fibrosis and liver architecture toward a normal pattern in less than 3 weeks, suggesting that massive and selective disruption of bile ducts and death of the newly formed bile duct epithelial cells rapidly occurred. That periportal fibrosis is resorbed after the relief of obstruction is sustained by a previous report showing a drop in the portal pressure following bilioduodenal anastomosis in extrahepatic cholestatic rats.[42] The reversibility of fibrosis is related to a dramatic decrease in the steady-state mRNA level for α_1 (I) and α_1 (IV) collagens and laminin B_2 chain. It is likely, however, that the disappearance of accumulated matrix proteins is mainly due to the activity of specific protease(s), including matrix metalloproteinases. The liver contains collagenase activity that generally augments during the early stage of liver injury,[43–45] and it has been shown that various liver cell types, including hepatocytes, fat-storing cells, and Kupffer cells, express collagenase activity *in vitro*.[46–48] The presence of collagenase has been demonstrated to be associated with fibrous septa in CCl_4-treated rat liver[49] and present in perinodular granulomas in murine Schistosomiasis.[50] In the latter situation, reversibility of liver fibrosis has been recently shown to parallel an increase in collagenase activity after treatment by a worm-targetted drug.[51] In cholestatic rats, the presence of a continuous layer of collagen IV in the sinusoids 3 weeks after restoration of bile outflow suggests that interstitial collagenase and collagenase IV are differently regulated in sinusoidal and/or parenchymal cells. A normal liver histology with the presence of a basement membranelike structure in the sinusoids is not unique. Similar features have been previously reported in human cases.[21,36] Further investigations are required to elucidate the mechanisms involved in the degradation of the different collagen types during the reversibility of hepatic fibrosis.

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Acknowledgments

The authors thank Drs. J. A. Grimaud (Institut Pasteur, Lyon, France), D. Louvard (Institut Pasteur, Paris, France), M. Laurent (INSERM U118, Paris, France), and Y. Yamada (NIDR, Bethesda, MD) for the gift of reagents. They also thank Drs. D. Ratanasavanh and Y. Cariou for their help in the enzyme assays.